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14. ABSTRACT

The purpose of this study was to determine the role of Notch in lymphatic endothelial cell (LEC) behavior and to determine the effects on tumor vasculature upon Notch inhibition. We hypothesized that disrupting Notch activity may interfere with tumor (lymph)angiogenesis by disrupting expression and activity of EC genes. To that end, we have created a treatment agent known as Notch1 decoy (hN1DFc). Activation or inactivation of Notch changes the gene profile of LEC and changes their in vitro behavior. An orthotopic model of human breast cancer was established. These tumors are rich in Notch-positive vasculature and metastasize to the lungs and/or lymph nodes. Tumor studies showed that hN1DFc did not significantly inhibit tumor growth, vascularization, or metastasis in this model. However, this does not exclude the possibility that hN1DFc could be an effective tumor growth/vascularization/metastasis inhibitor in other tumor models. Therefore, further investigation will be necessary to determine the potential use of Notch inhibitors in tumors.

15. SUBJECT TERMS

lymphatic endothelial cell, lymphangiogenesis, angiogenesis, Notch, Notch1 decoy (hN1DFc)

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Introduction

The American Cancer Society (ACS)'s statistics predict that in 2013, 232,340 new cases of breast cancer would be discovered, and 39,620 women would die from breast cancer. Additionally, about 1 in 8 women are estimated to develop breast cancer in their lifetime. Because breast cancer is such a common disease, the demand is high for research leading to new therapies. Several factors are considered when determining prognosis in breast cancer, including the size of the tumor and more importantly, if it has spread from its original location to other parts of the body. In order for a tumor to grow, it needs a constant supply of oxygen and nutrients, which can be provided by the recruitment of blood vessels to the tumor, also known as angiogenesis. In order for a tumor to spread to sentinel lymph nodes and eventually other parts of the body, it needs to recruit blood vessels (angiogenesis), lymphatic vessels (lymphangiogenesis), or both. Therefore, it is clear that both angiogenesis and lymphangiogenesis are critical steps that need to occur for tumor progression.

The Notch signaling pathway is a cell-fate determining pathway that consists of 4 receptors (Notch 1, 2, 3, 4) and 5 ligands (Delta-like 1, 3, 4 and Jagged 1, 2). When a Notch receptor interacts with a ligand and is activated, a series of proteolytic cleavages release the intracellular domain of the receptor from the cell membrane, allowing it to translocate to the nucleus and act as a transcriptional regulator. It has been well-established that Notch is present and active in the blood vasculature. Our lab has demonstrated that Notch can regulate endothelial cell (EC) genes. We hypothesize that disrupting Notch activity may interfere with tumor angiogenesis and lymphangiogenesis by disrupting expression and activity of key genes necessary for EC biology. To that end, we have developed a class of treatment agents known as Notch1 decoys, which comprise the EGF-like repeats in the extracellular domain of human Notch 1. These agents can effectively bind Dll and/or Jag ligands, but has no signaling capabilities, as they are missing both its transmembrane and intracellular domains.

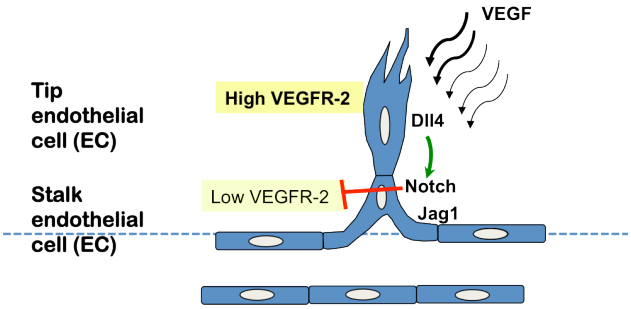
Body

Task 1. Study how Notch signaling functions in primary lymphatic endothelial cells

In the blood endothelium, Notch receptors 1 and 4, as well as ligands Dll-4 and Jag1, are present. Notch function in angiogenesis has already been well-established in the tip/stalk model (**Figure 1**). In this model, the tip endothelial cell, which is the leading cell in an angiogenic sprout, is exposed to high levels of vascular endothelial growth factor (VEGF), which leads to high expression of VEGF receptor 2 (VEGFR-2). High VEGFR-2 in the tip cell leads to high expression of the Notch ligand Dll-4. Dll-4 in the tip cell then signals to

the neighboring stalk endothelial cell, activating Notch in the stalk cell. Notch has been shown to inhibit expression of VEGFR-2 in the stalk cell, resulting in a cell that is less responsive to VEGF. This process ensures that there is just one cell – the tip cell – in an angiogenic sprout that is highly responsive to VEGF, thereby tightly regulating angiogenesis and allowing for proper formation of neovasculature. This has allowed for development of various Notch or ligand inhibitors, which can regulate angiogenesis by either inducing or inhibiting the process.

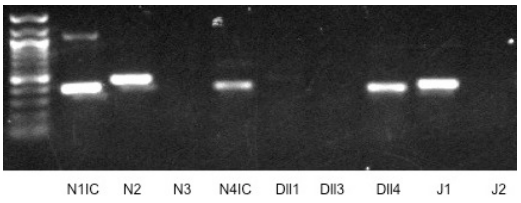
Figure 1 – Notch in the tip and stalk model



Next, we wanted to determine if Notch functions similarly in the lymphatic vasculature. We first decided to study Notch function in the behavior of primary lymphatic endothelial cells (LEC) *in vitro*. To do so, we established a method of isolating human dermal lymphatic endothelial cells (HDLEC) from human neonatal foreskins. Notch family receptors and ligands (Notch 1, Notch 2, Notch 4, Dll-4, Jag1) were present in our isolated HDLEC on the transcript **(Figure 2a)** and protein level **(Figure 2b)**.

Figure 2 – Notch receptors and ligands are present in primary lymphatic endothelial cells

a



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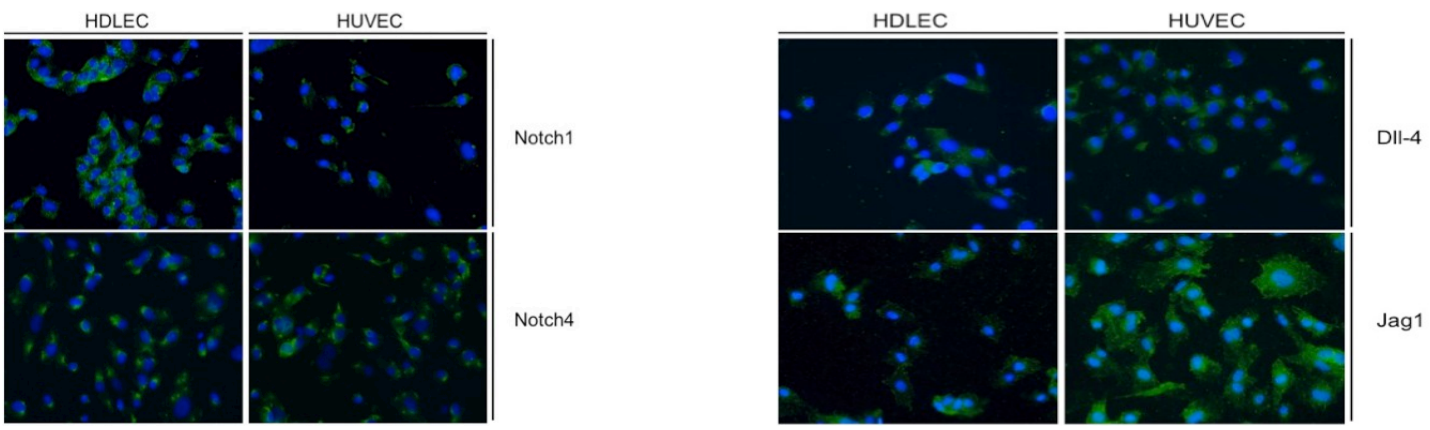
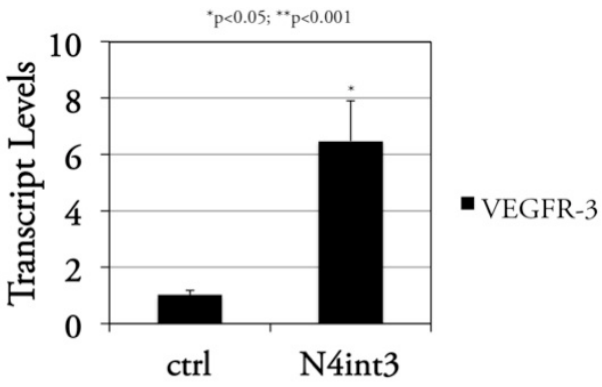


To activate Notch in HDLEC, lentiviral constructs expressing activated Notch 1 (N1IC) or activated Notch 4 (N4int3) were used. As expected, Notch activation (with either N1IC or N4int3) significantly induced transcripts for direct targets such as Hey1 and Hey2 (data not shown). VEGFR-3 was induced **(Figure 3a)**, while most other LEC genes (Prox1, LYVE1, and podoplanin) were repressed **(Figure 3b)**.

Figure 3 – Notch activation differentially regulates LEC genes

a



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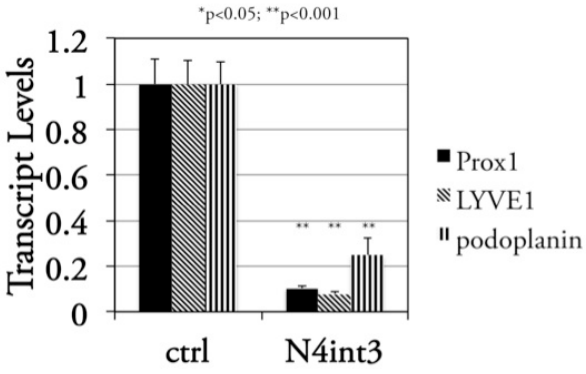
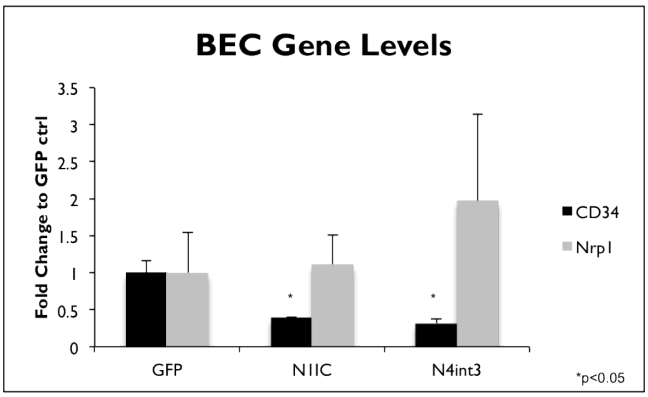


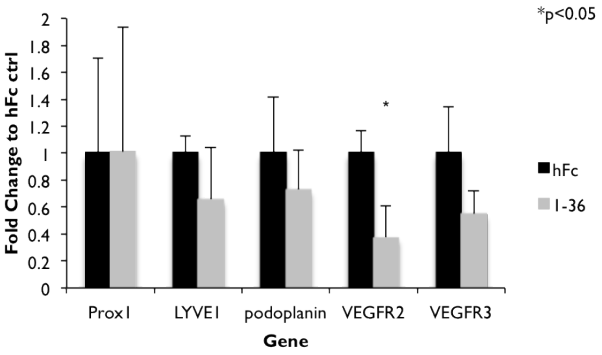
Upon observation that Notch activation repressed many LEC-associated genes, namely Prox1, which has been shown to be necessary to maintain LEC identity[1], we set out to determine whether Notch activation in HDLEC reverts LECs to BECs. Notch activation did not appear to revert LECs to BECs, as CD34 (a BEC marker) was repressed and Nrp1 (another BEC marker) was unaffected **(Figure 4)**.

Figure 4 – Notch activation does not revert HDLEC to BECs



To inactivate Notch in HDLEC, lentiviral constructs expressing Notch1 decoy (labeled 1-36 in below graph) or control (hFc) were used. Unexpectedly, Notch inactivation did not have any effect on LEC genes, but repressed VEGFR-2 **(Figure 5)**. To further confirm these results, it will be important to confirm inhibition of known Notch targets (e.g., Hey1 and Hey2) in the future. Additional studies to look at LEC genes in response to Notch inhibition are ongoing.

Figure 5 – Notch inhibition does not appear to affect LEC genes

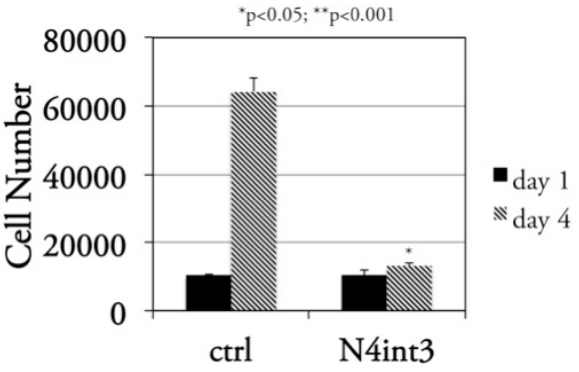


Effects of Notch activation on HDLEC behavior were tested in various different *in vitro* assays. Briefly, proliferation was tested by seeding equal numbers of cells for all conditions being tested, then quantifying cell number after 4 days of culture. Migration was observed by using a pipet tip to make equal-sized scratches in cell monolayers, then observing the migration of cells over time to close the scratch. Network formation was observed by plating equal numbers of cells in between two collagen gel layers and observing the formation of

networks over 4 days of culture. All assays were performed in serum-free endothelial media supplemented with EGF and VEGF-C. Results suggest that Notch activation in HDLECs inhibits *in vitro* proliferation, migration, and network formation **(Figure 6a, b, c)**.

Figure 6 – Notch activation inhibits HDLEC proliferation, migration, and network formation in vitro

a



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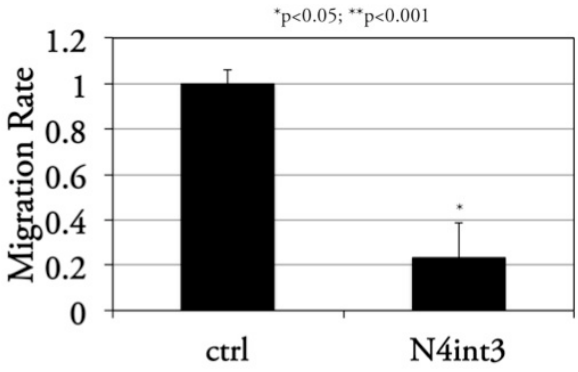

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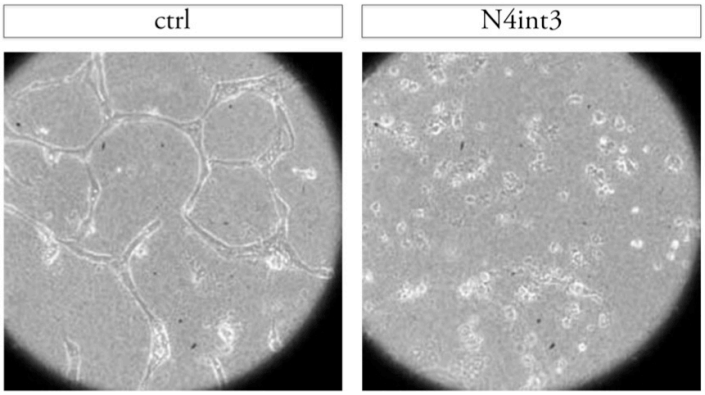
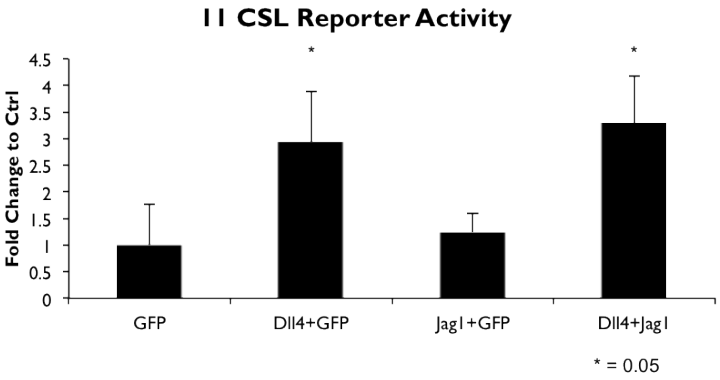


To determine whether Dll-4 and/or Jag1 are important in mediating Notch signaling in HDLEC, we performed a Notch reporter assay. Briefly, HDLEC were transfected with a Notch reporter (11-CSL) and co-cultured with HeLa cells (which do not endogenously express Notch receptors and ligands) transfected to overexpress Dll-4, Jag1, or both. 24 hrs after co-culture, a reporter assay was performed **(Figure 7)**. In this assay, Dll-4 was able to induce Notch reporter activity in HDLEC, while Jag1 did not significantly induce reporter activity. Additionally, Dll-4 and Jag1 together only induced Notch reporter activity to the level of Dll-4 alone. These results together suggest that in HDLEC, Dll-4 may be the dominant ligand that induces Notch signaling. However, these results need to be confirmed with replicates.

Figure 7 – Dll-4 can induce Notch signaling in HDLEC in vitro

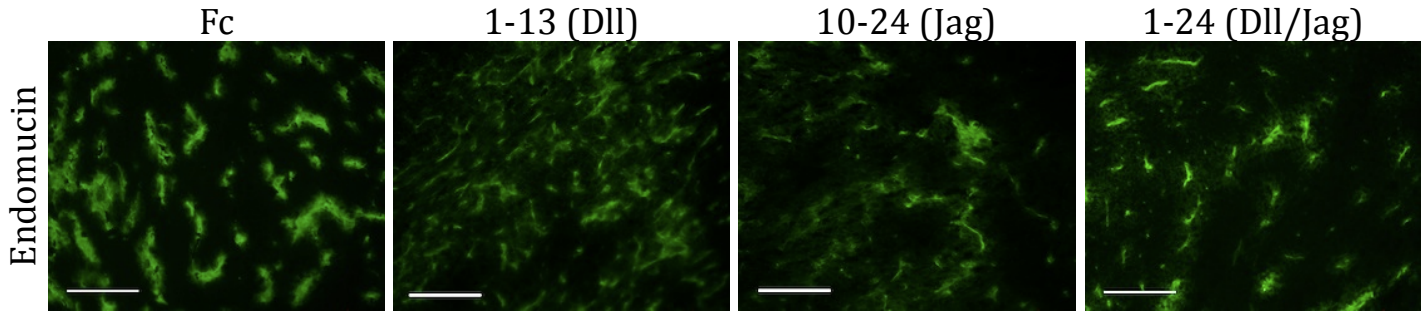


Taking all the *in vitro* results together, we are beginning to see that not only are Notch receptors and ligands present in lymphatic vasculature, but it is regulating LEC gene transcription and LEC behavior. These results strongly suggest that *in vivo* use of Notch inhibitors should have an effect on tumor blood and lymphatic vasculature.

Tasks 2 and 3. Study Notch function in pathological angiogenesis and lymphangiogenesis

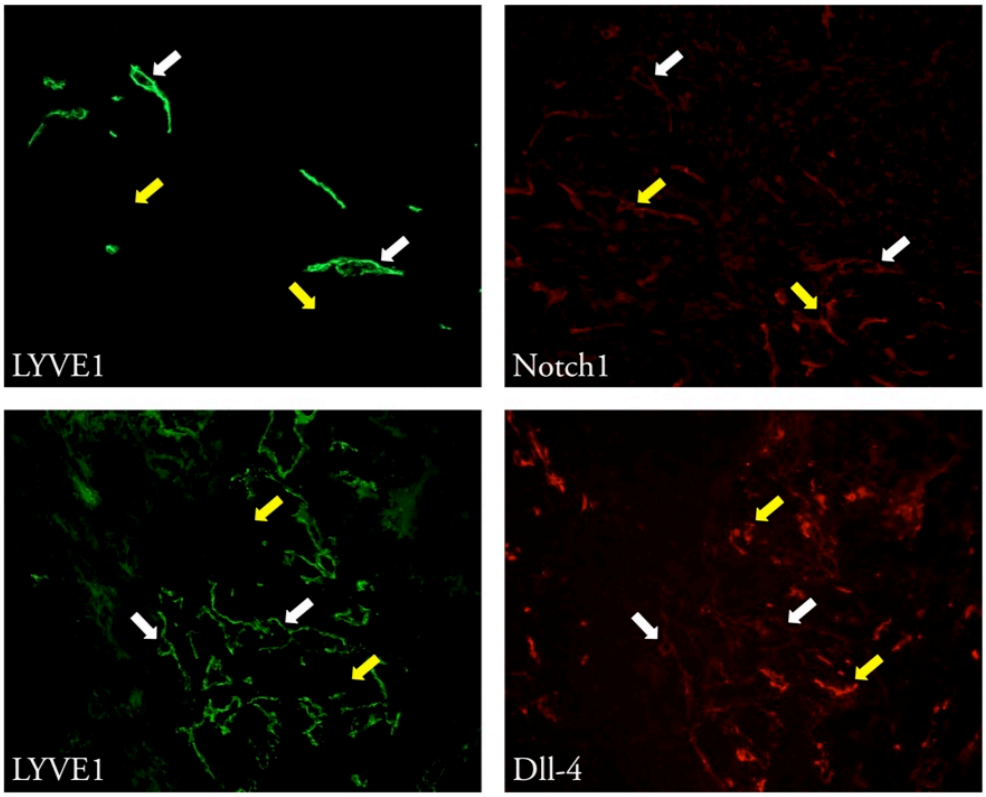
Previous tumor studies performed in our lab using several different xenograft models (Mm5MT mouse mammary tumor cells, KP1 pancreatic tumor cells, LLC Lewis Lung Carcinoma cells) had shown that Notch1 decoy could effectively shrink tumors (data not shown) through affecting tumor angiogenesis **(Figure 8, unpublished data)**. In these studies, the Notch1 decoy 1-24 (pan-ligand inhibitor), as well as decoy variants created to inhibit specifically Dll binding to Notch (containing EGF-like repeats 1-13) or Jag binding to Notch (containing EGF-like repeats 10-24) were able to shrink tumors by either repressing tumor angiogenesis (1-24, 10-24) or by inducing non-productive angiogenesis (1-13). This served as the basis for our motivation to study Notch1 decoy in an orthotopic model of breast tumor (lymph)angiogenesis.

Figure 8 – Notch decoy variants can differentially affect tumor angiogenesis



To study the effects of Notch inhibition in breast tumor vasculature *in vivo*, the MDA-MB-231 human breast cancer cell line was used for orthotopic tumor studies in nude mice. We initially obtained the subline MDA-MB-231-D3H1, which stably expresses luciferase, described previously by Xenogen[2]. Luciferase activity was useful for live imaging of tumor progression throughout the course of tumor studies. A survey revealed that transcripts for various Notch receptors/ligands, as well as VEGFR-2, were present in D3H1 (summarized in annual reports 1 and 2). Immunohistochemistry of pilot D3H1 tumors grown in the mammary fat pads of female nude mice demonstrated that Notch family receptors and ligands were indeed expressed by the tumors themselves, as well as in the blood (yellow arrows) and lymphatic (white arrows) vasculature **(Figure 9)**.

Figure 9 – D3H1 tumor vasculature expresses Notch1 and Dll4



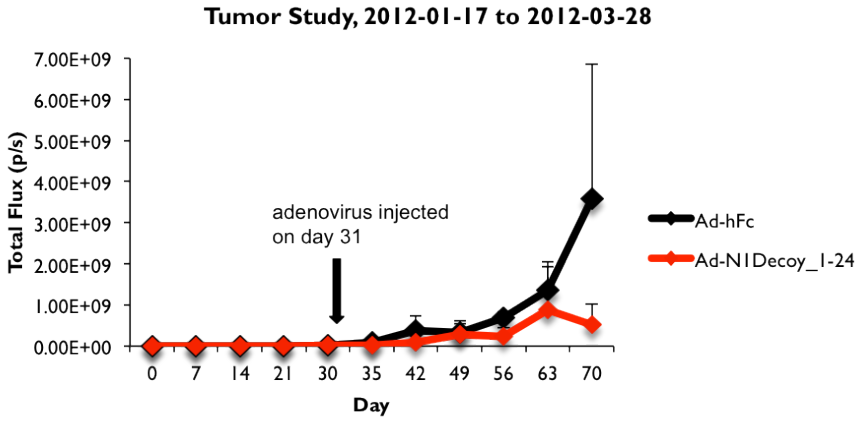
The D3H1 subline was used to first create a stable line that secretes VEGF-C C156s[3] (D3H1/Vc156s), a mutant VEGF-C with a point mutation (C156S) that confers specificity in binding and activation to VEGFR-3. D3H1's endogenously secrete VEGF-C, which can bind both VEGFR-2 and 3. Given our lab's previous data[4] that suggested Notch can directly affect VEGFR-3 expression in cultured LECs, we wanted to enrich VEGFR-3^{+} vasculature in our tumors, in order to study how our Notch inhibitor affects this vasculature.

D3H1/Vc156s were tested relative to control (D3H1/GFP) to determine whether *in vitro* proliferation or migration were affected by Vc156s. There were no significant differences in proliferation or migration (discussed in annual reports 1 and 2). Subsequently, pilot tumor studies were performed. In the first pilot study, we showed that Vc156s conferred a slight growth advantage over control (summarized in annual reports 1 and 2). *Ex vivo* imaging of lungs and lymph nodes found no difference between groups for lung metastases, but a significant difference between groups for metastases to axillary lymph nodes (summarized in annual reports 1 and 2). Immunostaining for CD31 (a general vessel marker that stains both blood and lymphatic vasculature) and LYVE1 (a lymphatic vessel marker) revealed that Vc156s induced tumor angiogenesis and lymphangiogenesis (summarized in annual reports 1 and 2). Thus, we were able to establish a useful model of human breast cancer, which induced robust tumor angiogenesis and lymphangiogenesis, as well as metastasis to lymph nodes, for future studies.

Subsequently, for our first full-scale tumor study, we tested the effects of our Notch inhibitor, Notch1 decoy on the growth and metastasis of the D3H1/Vc165s stable cell line (described in annual report 2). Briefly, D3H1/Vc156s tumors were implanted, and after 31 days, the mice were randomized into two treatment groups – Notch1 decoy or control (hFc). Treatment was administered via retro-orbital injection of adenovirus, which allowed for infection of the liver, resulting in the continued expression and secretion into the bloodstream of our treatment agent. In this round, Notch1 decoy treatment did not significantly affect tumor progression **(Figure 9a)**. However, average tumor weight of treated tumors trended towards being lower **(Figure 9b)**.

Figure 9 – Notch1 decoy does not affect D3H1/Vc156s tumor progression

a



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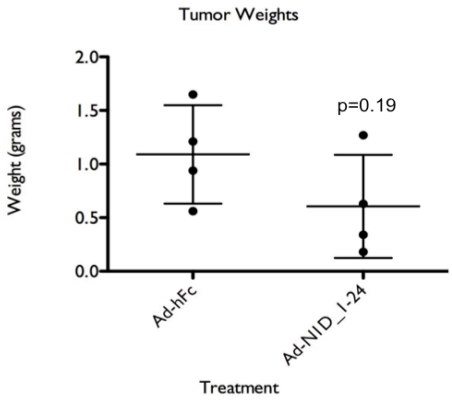


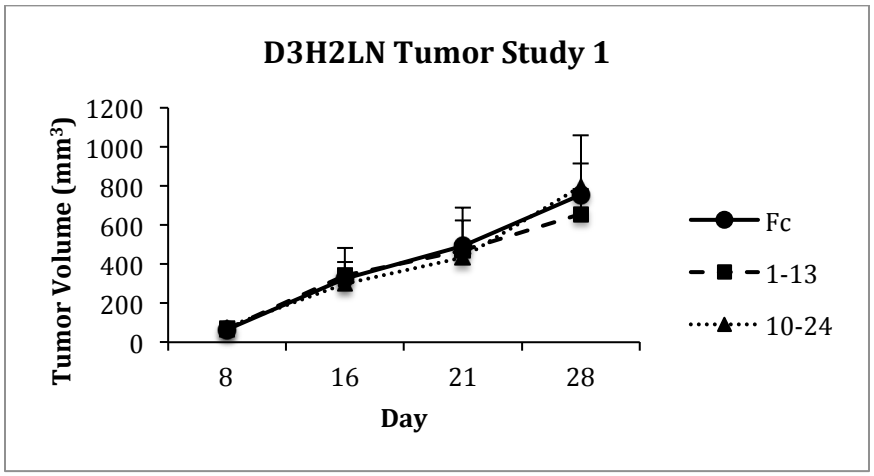
Subsequent immunostaining for LYVE1 (a lymphatic vessel marker) and endomucin (a blood vessel marker) suggested that Notch1 decoy treatment reduced tumor blood and lymphatic vessel density (summarized in annual report 2), but upon quantification was found not to be significant. Additionally, *ex vivo* imaging of lungs and lymph nodes proved to be challenging, whether this was due to the sensitivity of our detection capabilities, or due to the fact that the tumors were simply not metastasizing at the rate we had hoped for (summarized in annual report 2).

At this point, we revisited the original description of the D3H1 subline, and came across the D3H2LN subline[2]. According to the description, this subline seemed to be far better suited for our purposes (higher tumor take rate, shorter length of study, higher rate of metastasis to axillary lymph nodes). We performed a pilot study in which we compared our previous line (D3H1/Vc156s) to the new D3H2LN line, and found that the D3H2LN line did indeed have a better take rate, faster growth rate, and higher rate of metastasis (50% in our hands, though the publication observed 100% take rate[2]). All future experiments were performed using the D3H2LN subline.

In our first study using the D3H2LN line, $1.5*10^6$ D3H2LN cells were implanted into the 4^{th} mammary fat pad of 8 week old female nude mice. Adenoviruses encoding Notch1 decoy 1-13 (Dll inhibitor), 10-24 (Jag inhibitor), or control (Fc) were delivered through retro-orbital injection at day 8 and day 21 of the study, and serum was collected from each mouse weekly to validate expression of decoys throughout the study **(Figure 10b)**. As seen previously using the D3H1/Vc156s cell line, Notch1 decoys did not inhibit growth of D3H2LN tumors **(Figure 10a)**. Additionally, tumor weight **(Figure 10c)** and metastatic burden in axillary lymph nodes **(Figure 10d)** were unaffected. Summary of the study can be seen in **Figure 10e**.

Figure 10 – D3H2LN tumor progression unaffected by ligand-specific Notch decoys

a



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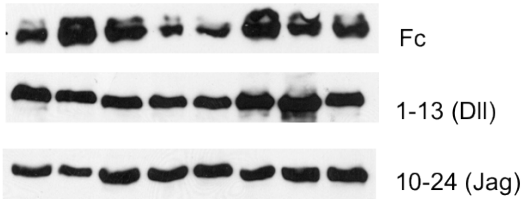

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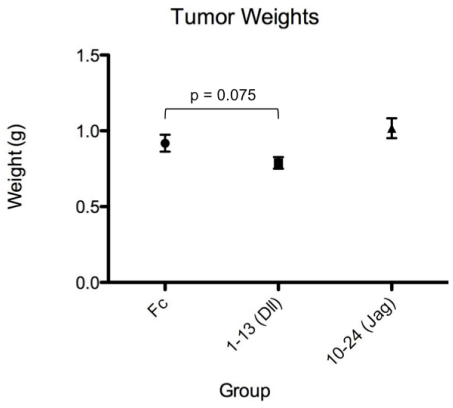

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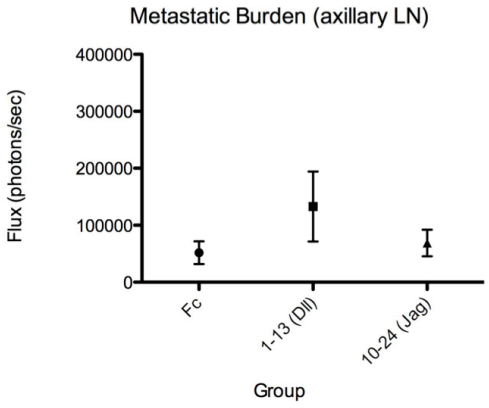


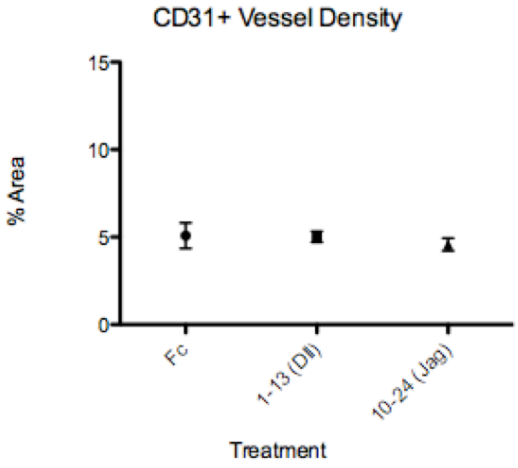
e

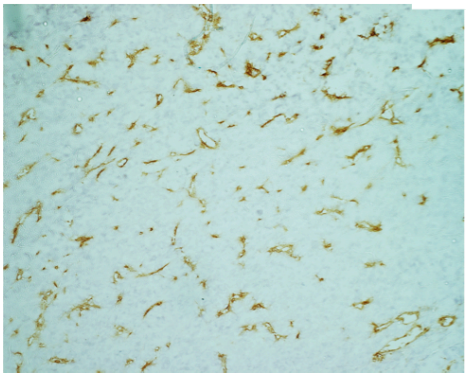
Group	Avg tumor luminescence day 28 (photons/sec)	Avg tumor volume day 28 (mm^3)	Avg mouse weight day 28 (g)	Axillary LN	Lungs
Fc (control)	3.48E+08	754.5170263	24.6625	5 of 8	0
1-13 (Dll)	2.91E+08	655.8546759	24.225	4 of 8	1 of 8
10-24 (Jag)	2.56E+08	796.4422052	25.1875	5 of 8	0

Furthermore, subsequent immunostaining for CD31 **(Figure 11a)**, LYVE1 **(Figure 11b)**, or F4/80 **(Figure 11c)** showed that Notch1 decoy treatment did not affect tumor blood vessel density or lymphatic vessel density, respectively.

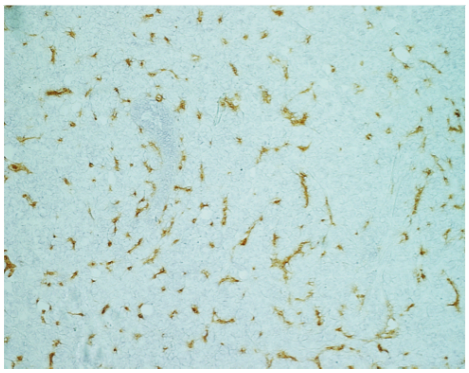
Figure 11 – D3H2LN tumor vasculature unaffected by ligand-specific Notch decoys

a

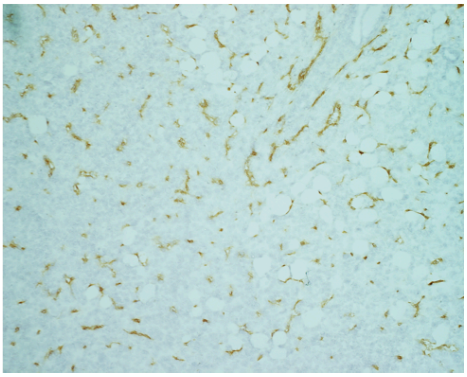




Fc

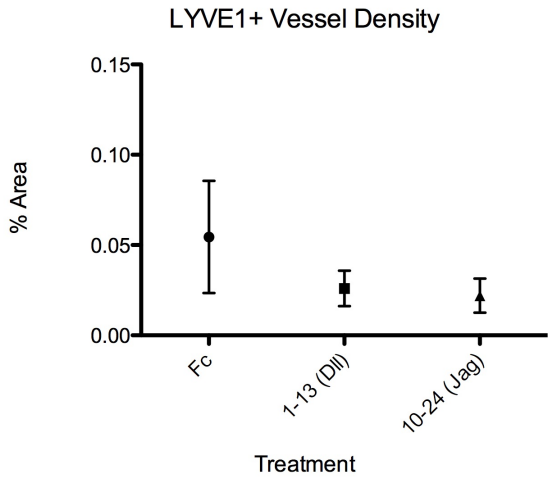


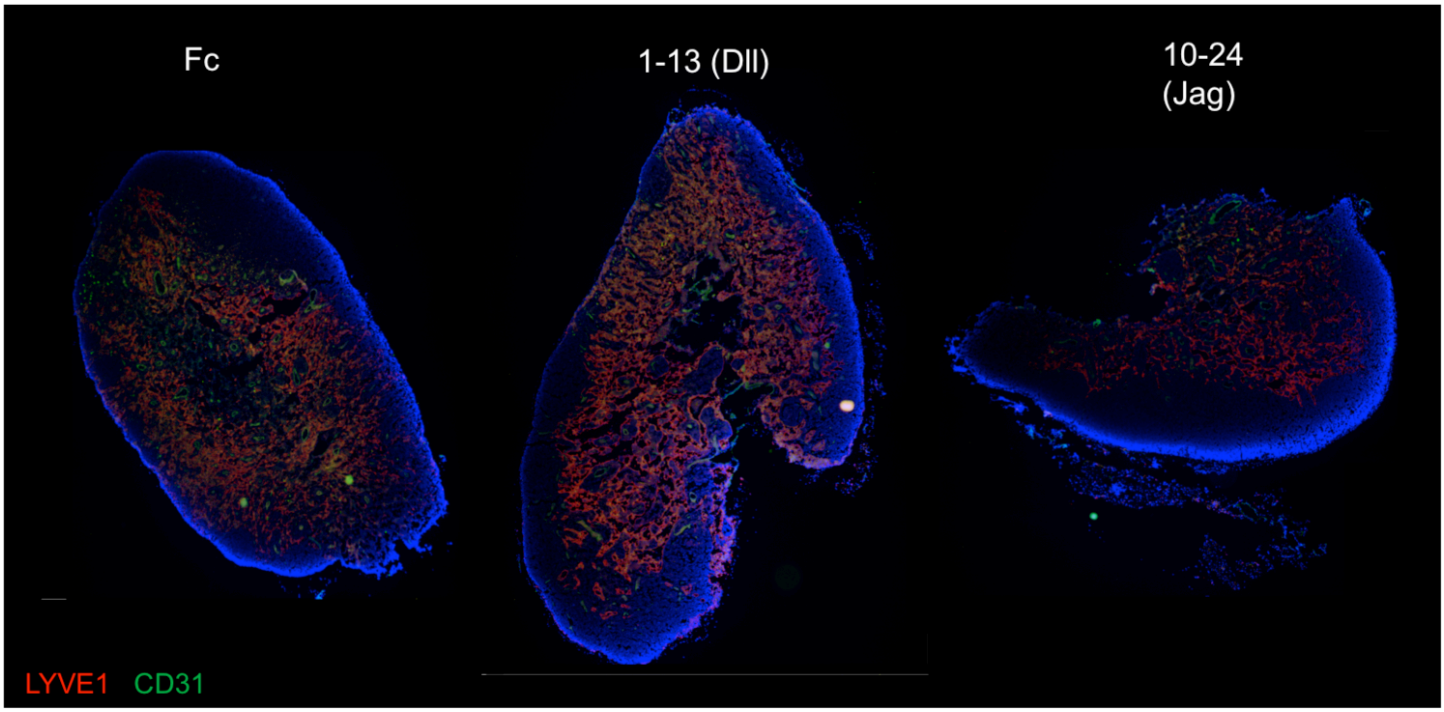
1-13 (Dll)



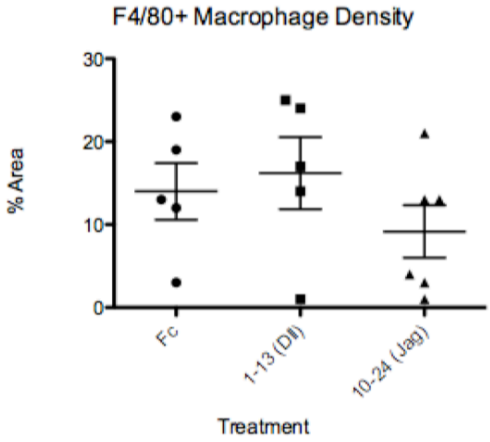
10-24 (Jag)

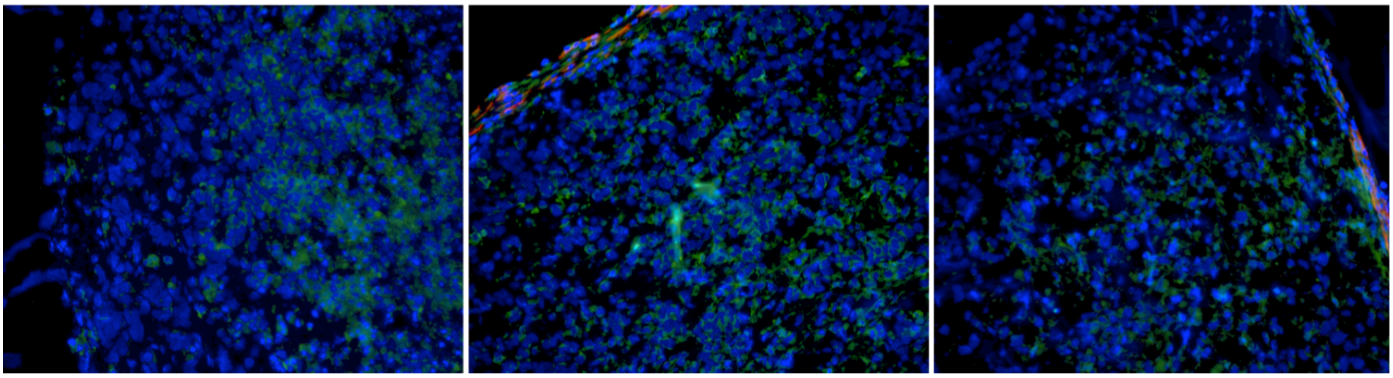
b


LYVE1+ Vessel Density
0.15
0.10
0.05
0.00
% Area
Fc
1-13 (Dll)
10-24 (Jag)
Treatment


Fc
1-13 (Dll)
10-24
(Jag)
LYVE1 CD31

c

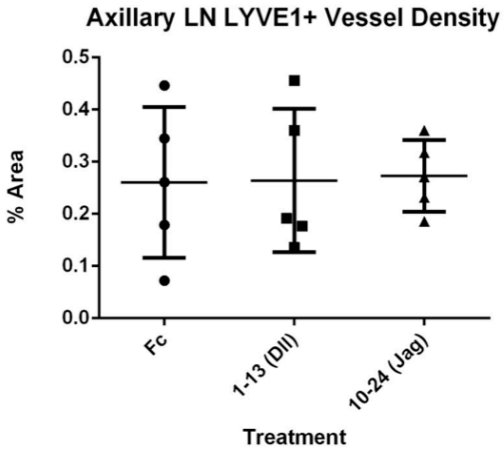


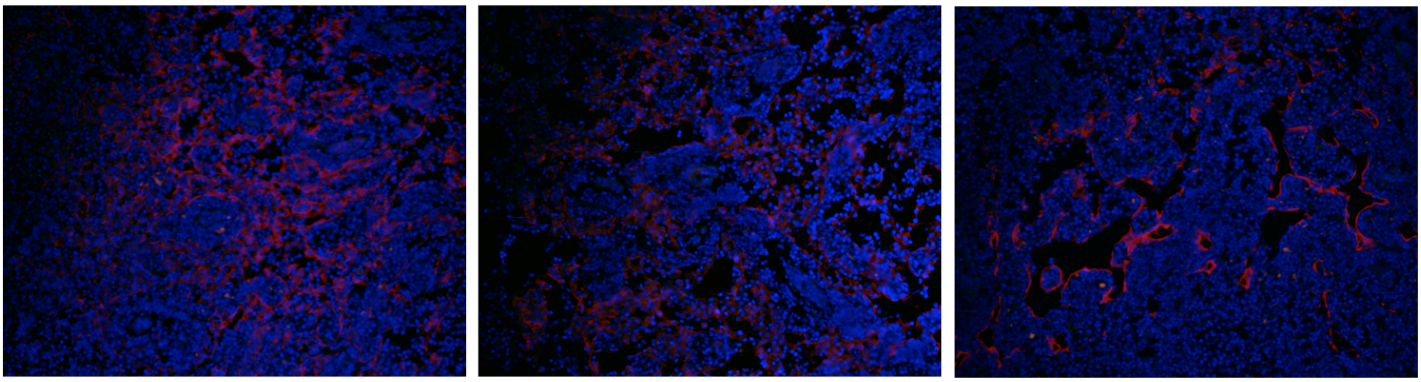


Fc 1-13 (Dll) 10-24 (Jag)

Axillary lymph node sections were also immunostained with LYVE1 to look at lymphatic vessel density. This too, was found to be unchanged among treatment groups **(Figure 12)**.

Figure 12 – Lymphatic vasculature in axillary lymph nodes of D3H2LN tumor bearing mice unaffected by ligand-specific Notch decoys





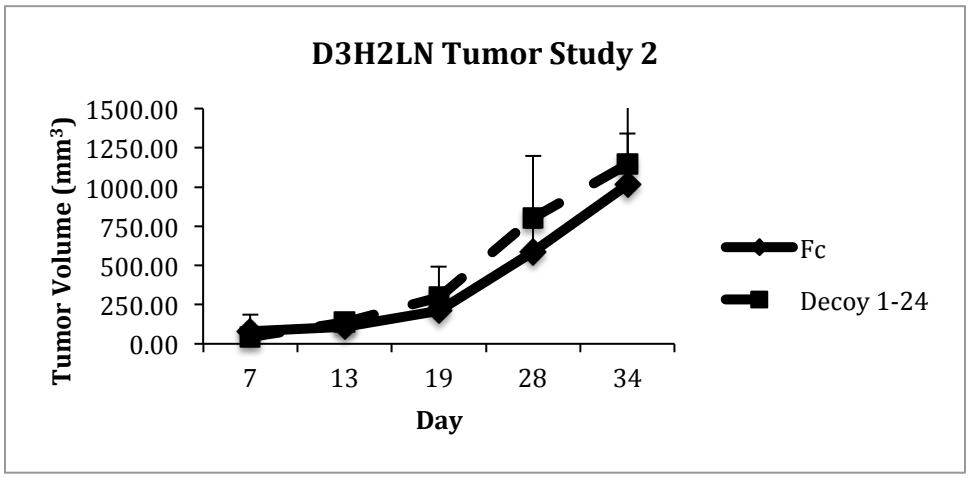
Fc 1-13 (Dll) 10-24 (Jag)

Although our Dll and Jag inhibiting Notch1 decoys did not have any effects on the parameters we studied, we hypothesized that it was possible that our 1-24 Notch1 decoy, which can inhibit both Dll and Jag mediated Notch signaling, could be more potent. Thus, for our second tumor study, we compared the effects of Notch1 decoy 1-24 with control (Fc). $5*10^5$ D3H2LN cells were implanted into the 4^{th} mammary fat pad of 8 week old female nude mice. Adenoviruses encoding Notch1 decoy 1-24 (Dll and Jag inhibitor) or control (Fc) were delivered through retro-orbital injection at day 0 and day 19 of the study, and serum was collected from each mouse weekly to validate expression of decoys throughout the study **(Figure 12b)**. As seen previously,

Notch1 decoy 1-24 did not inhibit growth of D3H2LN tumors **(Figure 12a)**. Additionally, tumor weight **(Figure 12c)** and metastatic burden in axillary lymph nodes **(Figure 12d)** were unaffected. Summary of the study can be seen in **Figure 12e**.

Figure 12 – D3H2LN tumor progression unaffected by pan-ligand Notch decoy

a



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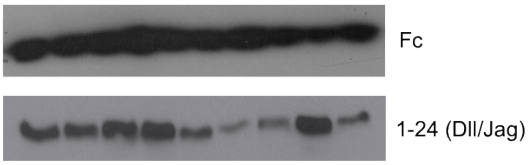

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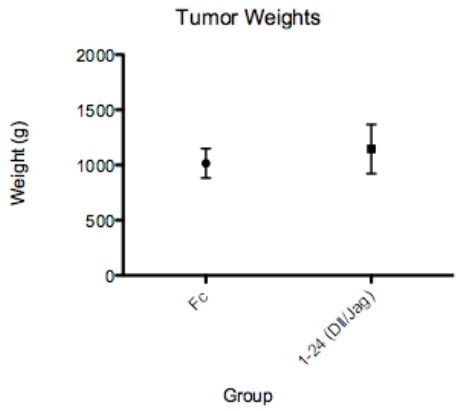

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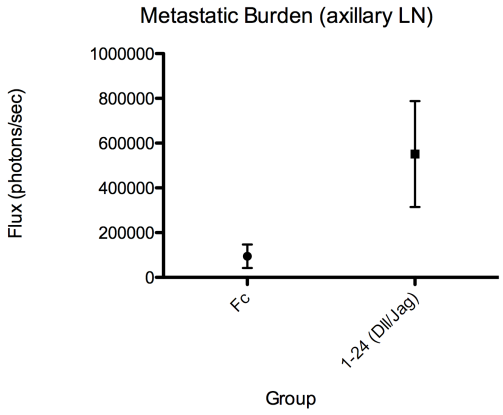


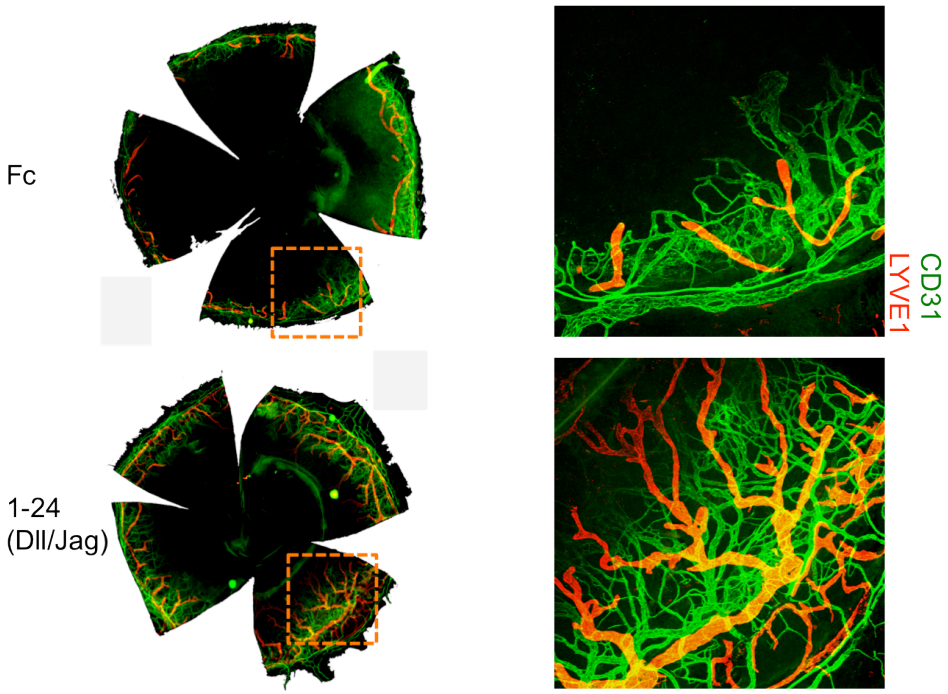
e

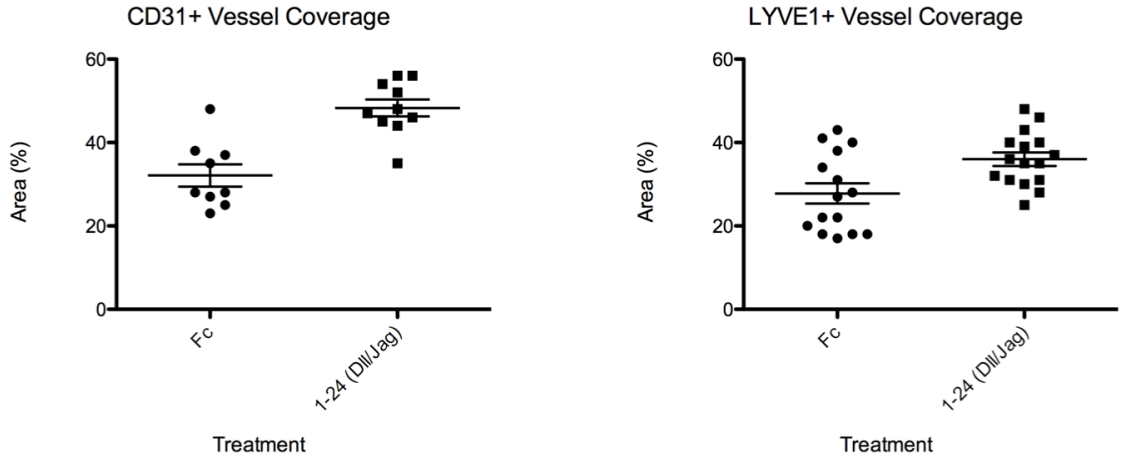
Group	Avg tumor luminescence day 34 (photons/sec)	Avg tumor volume day 34 (mm^3)	Avg mouse weight day 34 (g)	Axillary LN	Lungs
Fc (control)	1.86E+08	1015.319388	27.21666667	3 of 6	1 of 6
1-24 (Dll/Jag)	3.76E+08	1145.154935	28	3 of 5	1 of 5

Immunostaining for CD31, LYVE1, and F4/80 of both the primary tumors and axillary lymph nodes is currently in progress, but primary tumor growth analysis and metastasis rate using the IVIS imaging system suggests that Notch1 decoy treatment did not have an effect.

Although our proposed tumor studies did not show responsiveness to Notch1 decoy treatment, we continued to study Notch1 decoy's effects on other models of pathological angiogenesis and lymphangiogenesis. One such model that we have recently begun to study is the corneal suture model. In this model, a small nylon suture is placed in the cornea. This mounts an inflammatory response as macrophages come in to the site of the "wound", releasing various (lymph)angiogenic factors and inducing lymphangiogenesis and angiogenesis from pre-existing resting limbal vasculature into the normally avascular cornea over the course of 2 weeks. We tested our Notch1 decoy in this model, using 12 week old wild-type female C57BL/6J mice. Adenoviruses encoding Notch1 decoy 1-24 (Dll and Jag inhibitor), or control (Fc) were delivered through retro-orbital injection at day 0 or 4 of the study. Notch1 decoy 1-24 significantly induced pathological angiogenesis and lymphangiogenesis in the cornea **(Figure 13)**.

Figure 13 – pan-ligand Notch decoy induces pathological angiogenesis and lymphangiogenesis in the corneal suture model





*p<0.01 **p<0.005

Key Research Accomplishments

- Established a method of isolating primary human dermal lymphatic endothelial cells
- Demonstrated that Notch receptors and ligands are present in lymphatic endothelial cells (LEC)
- Demonstrated that perturbation of Notch signaling in LEC changes the gene profile of LEC
- Demonstrated that perturbation of Notch signaling in LEC inhibits proliferation, migration, and network formation *in vitro*
- Demonstrated that Notch receptors and ligands are present both in 231-luc tumors, as well as in their vasculature
- Established an orthotopic model of human breast cancer that recruits blood and lymphatic vasculature and metastasizes to lungs and lymph nodes (D3H1/Vc156s). This will be a good model to study how Notch inhibition affects tumor blood and lymphatic vessels.
- Demonstrated that secretion of a mutant VEGF-C (V_c156s) by 231-luc induces tumor lymphangiogenesis and increases metastasis to lymph nodes
- Established a new model with the D3H2LN subline
- Studies indicate that Notch1 decoy, used in its current form, does not inhibit tumor (lymph)angiogenesis, tumor growth, or tumor metastasis.

Reportable Outcomes

- Poster presentation for the Era of Hope DOD Breast Cancer Research Program Meeting 2011
- Master of Philosophy, Columbia University, awarded in October 2010
- Poster presentation for the Yale/NAVBO Lymphatic Circulation in Health and Disease Meeting 2013

Conclusion

I have demonstrated that Notch is expressed by isolated HDLEC *in vitro*. I have also shown that Notch plays a role in LEC behavior, as inducing Notch signaling affects HDLEC behavior *in vitro*. Induction of Notch signaling also changes the endothelial cell gene profile of HDLEC on the transcriptional level. Though the link between the changes in HDLEC gene profile and changes in *in vitro* activity has not yet been shown, my data suggests that induction of VEGFR-3 by Notch is not the sole factor in Notch's effects on HDLEC *in vitro*

behavior. Notch's ability to repress other EC genes such as Prox1, LYVE1, podoplanin, and VEGFR-2 may also play an important role. In future studies, it will be important to more thoroughly study the effects of Notch inhibition in HDLEC. It is interesting to note that preliminary results show that Notch inhibition does not have the opposite effects of Notch activation. This suggests that perturbation of Notch signaling in either direction (exogenously activating or inactivating signaling) may have similar effects on HDLEC gene expression and *in vitro* behavior.

Additionally, I was able to establish an orthotopic model of human breast cancer that recruits both blood and lymphatic vasculature and metastasizes to lungs and lymph nodes. I have shown that these tumors have Notch-positive blood and lymphatic vasculature. In speculating why Notch1 decoy did not have the expected effect on repressing tumor (lymph)angiogenesis, growth, and metastasis, there are several possibilities. One possibility is that some factor in the mammary fat pad microenvironment is pro-tumor growth for our orthotopically implanted breast tumor cells, and that this overcomes the capability of the Notch1 decoy to inhibit tumor (lymph)angiogenesis, growth, and metastasis. All previous studies were performed as subcutaneous xenografts, whether they were mammary tumors, pancreatic tumors, lung carcinoma tumors, etc. Another possibility is that we have not yet achieved the levels of Notch1 decoy necessary in the mouse to see real changes. Our current method of delivering Notch1 decoy is not well-controlled, as we can only estimate steady-state levels of Notch1 decoy proteins in the circulation through Western Blot. It may be necessary to use purified Notch1 decoy proteins and perform dosing studies to determine maximum tolerated dose, median effective dose, etc. Additionally, it may be that Notch1 decoy treatment must be administered in combination with other anti-(lymph)angiogenic or chemotherapeutic agents. Finally, we can not rule out the possibility that Notch1 decoys are not ideal reagents for use as anti-(lymph)angiogenic agents. However we believe that there is still much to investigate using Notch1 decoys in the future.

In the cornea, Notch1 decoy had the opposite effect of what was expected and of what we have observed in the tumor – induction of lymphangiogenesis and angiogenesis instead of inhibition. However, these results suggest that Notch1 decoy can affect pathological (lymph)angiogenesis, which provides further motivation to continue studying Notch1, either using Notch inhibitors like our Notch1 decoy, or Notch activations, like N1IC or N4int3, as potential tumor (lymph)angiogenic inhibitors.

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PEER REVIEWED RESEARCH ARTICLES

1. Murtomaki, A., **Uh, M.K.**, Zhao, J., Nagasaki, T., Shawber, C.J., Kitajewski, J. (2013) Notch signaling regulates lymphatic valve formation. (*in preparation*).
2. Murtomaki, A.*, **Uh, M.K.***, Choi, Y.K., Kitajewski, C., Borisenko, V., Kitajewski, J., Shawber, C.J. (2013) Notch1 functions as a negative-regulator of lymphatic endothelial cell differentiation in the venous endothelium. *Development,* **140(11):** 2365-76.
3. Schmitz, Y., Luccarelli, J., **Kim, M.**, Wang, M., and Sulzer, D. (2009) Glutamate Controls Growth Rate and Branching of Dopaminergic Axons. *Journal of Neuroscience,* **29(38):** 11973-11981.

BOOK CHAPTERS

1. Uh, M.K., Kandel, J., Kitajewski, J. *Evaluating Tumor Angiogenesis*. 2nd ed. 980. New York: Springer, 2013. 341-51. Print.